278. *Nucleotides. Part XLIV.* Thymidine-5′ Pyrophosphate and Triphosphate.*

By Mrs. B. E. Griffin and Sir Alexander Todd.

Thymidine-5′ pyrophosphate has been obtained in 70% yield starting from 3′-*O*-benzylthymidine by converting it into 3′-*O*-benzylthymidine-5′ benzyl phosphorochloridate, condensing this with a salt of benzyl dihydrogen phosphate, and subsequently removing protecting groups. Thymidine-5′ triphosphate was similarly prepared in over 40% yield from the same phosphorochloridate by reaction with a salt of P^1P^2-dibenzyl pyrophosphate, followed by debenzylation.

The importance of deoxyribonucleoside polyphosphates in certain biological systems [1] makes their preparation by chemical methods desirable, partly to aid in the characterisation of the naturally occurring materials and more particularly to render them more available

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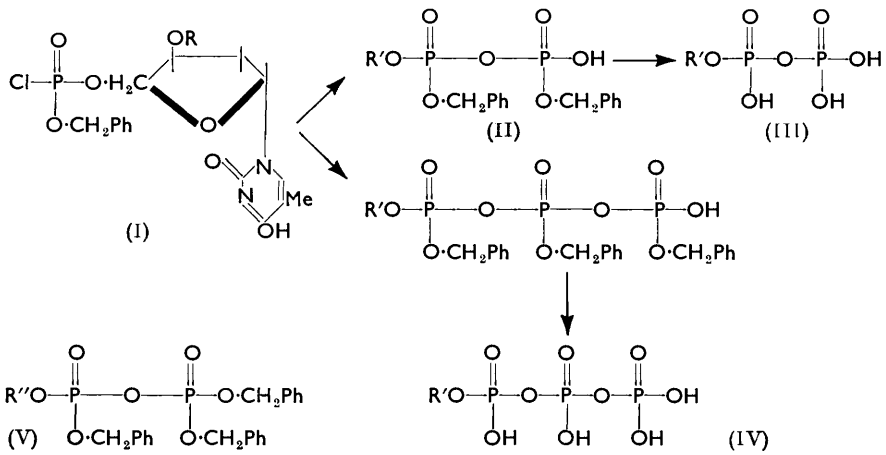
[1] Potter and Schlesinger, *J. Amer. Chem. Soc.*, 1955, **77**, 6714; Klenow and Lichtler, *Biochim. Biophys. Acta*, 1957, **23**, 6.

for use in the study of enzyme systems. We now report one stage of our investigations in this field, namely, convenient preparations of thymidine-5′ pyrophosphate (III; R′ = thymidine-5′) and triphosphate (IV; R′ = thymidine-5′); we believe them to represent an improvement on those employing carbodi-imides.[2]



In endeavouring to prepare thymidine-5′ pyrophosphate we first applied the method used by Kenner, Todd, and Weymouth[3] in their synthesis of uridine-5′ pyrophosphate (UDP). 3′-*O*-Acetylthymidine-5′ benzyl phosphorochloridate (I; R = Ac), prepared from the known 3′-acetylthymidine,[4] was condensed with a salt of dibenzyl hydrogen phosphate and the product was debenzylated by short treatment with *m*-cresol,[5] followed by hydrogenolysis. Unfortunately, removal of the residual acetyl group under alkaline conditions was accompanied by considerable breakdown of the pyrophosphate linkage and the yield of thymidine-5′ pyrophosphate (III; R′ = thymidine-5′) was low. Since benzyl ethers of nucleosides can be cleaved by hydrogenation,[6] we sought to improve the preparation by using 3′-*O*-benzylthymidine (prepared from 5′-*O*-tritylthymidine by benzylation and removal of the trityl residue) in place of 3′-*O*-acetylthymidine in the above preparation. Debenzylation was effected as before by treatment with *m*-cresol followed by hydrogenation; a somewhat higher yield (*ca.* 25%) of thymidine-5′ pyrophosphate was obtained, but the crude product contained in addition large quantities of thymidine-5′ phosphate and inorganic pyrophosphate which made purification by ion-exchange chromatography very difficult. These by-products undoubtedly arise from reaction of the fully esterified initial product (V; R″ = 3′-*O*-benzylthymidine-5′) with dibenzyl phosphate anion, leading to the formation of 3′-*O*-benzylthymidine-5′ benzyl phosphate and tetrabenzyl pyrophosphate. Such exchange reactions of fully esterified pyrophosphates are well known and an example of a similar type has recently been discussed by Kenner, Reese, and Todd[7] in connection with the synthesis of P^1-adenosine-5′ P^2-uridine-5′ pyrophosphate.

These findings suggested that better results in syntheses of this nature might be achieved by using salts of monobenzyl phosphate rather than of dibenzyl phosphate in the initial reaction since the product would be only a triply esterified pyrophosphate of type (II), and, as such, less likely to undergo an exchange reaction leading to ultimate production of inorganic pyrophosphate. This proved to be so, and condensation of 3′-*O*-benzylthymidine-5′ benzyl phosphorochloridate (I; R = CH_2Ph) with benzyl triethylammonium

[2] Potter, Schlesinger, Buettner-Janusch, and Thompson, *J. Biol. Chem.*, 1957, **226**, 381.
[3] Kenner, Todd, and Weymouth, *J.*, 1952, 3675.
[4] Michelson and Todd, *J.*, 1953, 951.
[5] Kenner, Todd, Webb, and Weymouth, *J.*, 1954, 2288.
[6] Michelson and Todd, *J.*, 1956, 3459.
[7] Kenner, Reese, and Todd, *J.*, 1958, 546.

hydrogen phosphate, followed by debenzylation, gave thymidine-5′ pyrophosphate in 70% yield, uncontaminated by inorganic pyrophosphate and containing only traces of orthophosphate. Ion-exchange chromatography was unnecessary, the product being precipitated directly in a pure state as its calcium salt. It may be noted that Michelson and Todd [6] mention a preparation of uridine-5′ pyrophosphate (UDP) by the same type of reaction although as the experiment was carried out only to verify the nature of the phosphorochloridate used, no details of yield were given.

We then applied an analogous procedure to the preparation of thymidine-5′ triphosphate (IV; R = thymidine-5′). 3′-*O*-Benzylthymidine-5′ benzyl phosphorochloridate (I; R = CH_2Ph) was condensed with a salt of P^1P^2-dibenzyl pyrophosphate, and the product was debenzylated as before. The crude product, which contained some thymidine-5′ phosphate, thymidine-5′ pyrophosphate, and inorganic pyrophosphate, as well as the desired triphosphate, was purified by ion-exchange chromatography and isolated as barium thymidine-5′ triphosphate; the yield was 43%, based on 3′-*O*-benzylthymidine.

Experimental

Thymidine-5′ Pyrophosphate from 3′-O-*Acetylthymidine.*—A solution of 3′-*O*-acetylthymidine [4] (1 mol.) was treated with *O*-benzylphosphorous *OO*-diphenylphosphoric anhydride (2 mols.) as described by Kenner, Todd, and Weymouth,[3] and the crude phosphite purified by precipitation from chloroform solution; it was obtained as a yellow gum which on paper chromatography (Whatman No. 1 paper; saturated butan-1-ol–water; ascending chromatogram) showed a single ultraviolet-absorbing spot of R_F 0·70.

A mixture of this phosphite (0·67 g., 1 mol.) and *N*-chlorosuccinimide (0·21 g., 1 mol.) in benzene (15 c.c.) and methyl cyanide (1·5 c.c.) was set aside for 2 hr. at room temperature. Dibenzyl hydrogen phosphate (0·42 g., 1 mol.) and triethylamine (0·15 g., 1 mol.) were added; precipitation of triethylamine hydrochloride set in at once. The mixture was stirred for 3 hr., then washed successively with water, aqueous potassium hydrogen carbonate, N/50-hydrochloric acid, and again water, dried (Na_2SO_4), and evaporated to a straw-coloured oil. The oil was heated at 70° for 15 min. with *m*-cresol (3 c.c.), then cooled, water (20 c.c.) was added and *m*-cresol removed by ether-extraction. The aqueous solution was adjusted to pH 6—7 with 0·1N-barium hydroxide and hydrogenated (6 hr.) at room temperature and atmospheric pressure in presence of palladous oxide–palladised charcoal. Electrophoretic examination of the filtered solution in sodium acetate buffer (pH 4·8) indicated two main components, apparently the 5′-phosphate and rather more of the 5′-pyrophosphate of 3′-acetylthymidine.

Deacetylation was effected by adjusting the solution to pH 10 with barium hydroxide and maintaining it at this pH for 2—3 days. Paper chromatography (see below) showed two main ultraviolet-absorbing constituents corresponding to thymidine-5′ phosphate and pyrophosphate. By means of anion-exchange chromatography (as described below) thymidine-5′ pyrophosphate was obtained as a lithium salt (100 mg., 16%) which was free from other nucleotides but contained inorganic phosphate.

3′-O-*Benzylthymidine.*—Benzyl chloride (4·5 c.c.) and powdered potassium hydroxide (9 g.) were added to a solution of 5′-tritylthymidine [4] (3 g.) in benzene (30 c.c.) and dioxan (10 c.c.) and the mixture was refluxed for 4 hr. with vigorous stirring. Water (30 c.c.) was added to the cooled mixture, which was then neutralised with acetic acid. The benzene layer was separated, washed with water, dried (Na_2SO_4), and evaporated. The crude oily product was twice redissolved in benzene and precipitated with light petroleum (b. p. 60—80°) before being refluxed for 10 min. with aqueous 80% acetic acid (15 c.c.) to remove the 5′-trityl group. The acetic acid solution was then set aside at room temperature for several hours. Precipitated triphenylmethanol was removed and washed with cold aqueous 80% acetic acid (5 c.c.), and the combined filtrate and washings were poured into ice-water (300 c.c.). The milky solution was concentrated *in vacuo* below 30°; colourless needles began to be formed when about half of the liquid had been removed. Evaporation was continued until the bulk of solution was reduced to 15 c.c., then the crystalline product was collected, washed with cold water, and dried. Recrystallised from acetone–*n*-heptane, 3-O-*benzylthymidine* formed colourless needles (1·3 g., 64%),

m. p. 148—150° (Found: C, 61·6; H, 6·0; N, 8·4. $C_{17}H_{20}O_5N_2$ requires C, 61·4; H, 6·1; N, 8·4%).

Thymidine-5′ Pyrophosphate from 3′-O-Benzylthymidine.—(a) *Use of dibenzyl hydrogen phosphate.* 3′-*O*-Benzylthymidine (1 g.) was converted into its 3′-*O*-benzylthymidine-5′ benzyl phosphite as described above for the 3′-*O*-acetyl derivative, and the product was dissolved in benzene (20 c.c.) and methyl cyanide (2 c.c.). *N*-Chlorosuccinimide (0·37 g.) was added and the mixture set aside for 2 hr., moisture being excluded. A solution of dibenzyl hydrogen phosphate (0·78 g.) and triethylamine (0·4 c.c.) in benzene (10 c.c.) was added dropwise with stirring, and stirring was continued for a further 2 hr. The mixture was washed successively with water, potassium hydrogen carbonate solution, N/50-hydrochloric acid, and water, dried (Na_2SO_4), and evaporated to a thick oil. The oil was partially debenzylated with *m*-cresol as described above and debenzylation was completed by hydrogenation at room temperature and atmospheric pressure in aqueous solution at pH 3·5—4 (palladium black). The resulting solution (50 c.c.) was adsorbed on a column of anion-exchange resin (Dowex-2, chloride form; 10 × 1·5 cm.). Thymidine-5′ phosphate was eluted with 0·01N-hydrochloric acid, and 0·01N-hydrochloric acid containing lithium chloride (0·1M) eluted thymidine-5′ pyrophosphate free from other nucleotidic material (paper chromatography). The eluted thymidine-5′ pyrophosphate was isolated as lithium salt (315 mg., 25%) but paper chromatography showed that it contained a considerable amount of inorganic pyrophosphate (molybdate spray).[8] To remove inorganic pyrophosphate the crude lithium salt (80 mg.) was converted into the barium salt, then precipitated under controlled pH conditions as described for the analogous uridine-5′ pyrophosphate.[9] Barium pyrophosphate was precipitated below pH 4, and after adjustment to pH 6 *barium thymidine-5′ pyrophosphate* was precipitated as a white powder by addition of ethanol (5 vol.). Several such precipitations were necessary to obtain the barium salt (40 mg.) free from inorganic material (Found in material dried at room temperature/1 mm. over P_2O_5: C, 18·2; H, 3·4; N, 4·3; total P, 9·5, 9·7; acid-labile P, 4·8. $C_{10}H_{13}O_{11}N_2P_2Ba_{1·5},3H_2O$ requires C, 18·2; H, 2·9; N, 4·3; total P, 9·4; acid-labile P, 4·7%).

(b) *Use of benzyl dihydrogen phosphate.* To a solution of 3′-*O*-benzylthymidine-5′ benzyl phosphorochloridate (prepared as described above from 0·16 g. of 3′-*O*-benzylthymidine) in benzene (10 c.c.) and methyl cyanide (1 c.c.), benzyl dihydrogen phosphate [10] (87 mg.) and triethylamine (0·13 c.c.) were added. The mixture was stirred for 2 hr., filtered from triethylamine hydrochloride, and evaporated. The thick oil obtained was examined by paper chromatography in solvent system A (see below) and showed a single ultraviolet-absorbing spot (R_F 0·89). A solution of the oil in water (20 c.c.) was brought to pH 3·4 with acetic acid and hydrogenated (16 hr.) at room temperature and atmospheric pressure (palladium black). Catalyst was filtered off and the filtrate (which appeared to contain thymidine-5′ pyrophosphate as the sole ultraviolet-absorbing constituent) was brought to pH 6 with aqueous lithium hydroxide; the lithium salt of thymidine-5′ pyrophosphate (100 mg.) was precipitated as a white powder by addition of acetone (100 c.c.). Evaporation, to small bulk, of the mother-liquor left after removal of this salt and addition of acetone yielded a further quantity (35 mg.) of product of similar purity. The yield of the lithium salt at this stage was *ca.* 70% based on 3′-*O*-benzylthymidine and paper chromatography showed that although it contained a small amount of orthophosphate it was free from inorganic pyrophosphate.

For analysis *calcium thymidine-5′ pyrophosphate* was prepared from the lithium salt by dissolution in water (5 c.c.) and addition of hydrochloric acid (to pH 2) and then calcium chloride (60 mg.); a small amount of orthophosphate was removed by adding calcium hydroxide (to pH 6) and filtering, and the calcium salt was then precipitated from the filtrate by adding 3 : 1 acetone–ethanol (50 c.c.). The salt was washed first with acetone–ethanol, then ether, and dried at room temperature (Found, in air-dried material: C, 20·3; H, 3·8; N, 4·7; P, 10·4, 10·7. $C_{10}H_{13}O_{11}N_2P_2Ca_{1·5},7H_2O$ requires C, 20·5; H, 4·6; N, 4·8; P, 10·6%).

Thymidine-5′ Triphosphate.—Diammonium P^1P^2-dibenzyl pyrophosphate [11] was converted into the di(phenyltrimethylammonium) salt by passing an aqueous solution (30 c.c.) through a column of Dowex-50 ion-exchange resin (5 × 1·5 cm.) in the phenyltrimethylammonium form. Evaporation of the solution gave a thick oil which was dried by azeotropic distillation with

[8] Hanes and Isherwood, *Nature*, 1949, **164**, 1107.
[9] Anand, Clark, Hall, and Todd, *J.*, 1952, 3665.
[10] Chase, Kenner, Todd, and Webb, *J.*, 1956, 1371.
[11] Clark, Kirby, and Todd, *J.*, 1957, 1497.

benzene, then dissolved in methyl cyanide and filtered from a small amount of insoluble material. The salt, which was obtained as exceedingly hygroscopic needles by evaporating the methyl cyanide solution, was not analysed but used directly in the following experiment.

A solution of 3′-*O*-benzylthymidine-5′ benzyl phosphorochloridate [prepared from 3′-*O*-benzylthymidine (332 mg., 1 mol.) as described above] in benzene (10 c.c.) and methyl cyanide (1 c.c.) was added to solution of di(phenyltrimethylammonium) P^1P^2-dibenzyl pyrophosphate (940 mg., 1·5 mol.) in methyl cyanide (4 c.c.). The mixture was stirred at room temperature for 2 hr., then filtered, the residue was washed with 3 : 1 benzene–methyl cyanide (5 c.c.), and the combined filtrate and washings were evaporated *in vacuo* to a thick oil. Water (20 c.c.) was added, and the resulting solution brought to pH 3·5 with acetic acid and then hydrogenated (24 hr.) in the usual way with palladium black. Paper chromatography of the filtered hydrogenation solution showed that it contained as major solute a material with the properties expected of thymidine-5′ triphosphate together with some thymidine-5′ phosphate, inorganic pyrophosphate, and a trace of thymidine-5′ pyrophosphate. The solution (50 c.c.) was adjusted to pH 6 with ammonia and put on a column of Dowex-2 resin (chloride form; 8 × 2·5 cm.). Elution with 0·01N-hydrochloric acid containing lithium chloride (0·1M) removed most of the impurities, and the triphosphate was then eluted with 0·01N-hydrochloric acid containing a larger amount (0·2M) of lithium chloride. Elution was followed by examination of ultraviolet absorption, and appropriate fractions were combined, brought to pH 6 with lithium hydroxide, and concentrated to *ca.* 30 c.c. *in vacuo* below 30°. A concentrated aqueous solution of barium acetate (300 mg.) was then added, followed by ethanol (150 c.c.), and the mixture was set aside at 0° overnight. The separated barium salt was centrifuged off, washed with water (2 × 5 c.c.), to remove barium acetate, then with 1 : 1 aqueous ethanol (10 c.c.), finally with ethanol and ether, and dried. The white powder so obtained (363 mg., 43% based on 3′-*O*-benzylthymidine) ran as a single ultraviolet-absorbing spot on paper chromatography and contained only traces of inorganic pyrophosphate (molybdate spray) as impurity.

To obtain a sample for analysis, the above salt was dissolved in cold N-hydrochloric acid, and the solution brought to pH 3·5 with barium hydroxide. The precipitate contained all the inorganic impurity as well as a substantial amount of the triphosphate. It was filtered off and the filtrate brought to pH 6 with barium hydroxide and diluted with alcohol (3 vol.). The white precipitate of hydrated *barium thymidine-5′ triphosphate* was collected, washed with ethanol, then ether, and dried (Found, in air-dried material: C, 14·2; H, 2·7; N, 3·2; total P, 11·2; acid-labile P, 7·3. $C_{10}H_{13}O_{14}N_2P_3Ba_2,5H_2O$ requires C, 14·2; H, 2·8; N, 3·3; total P, 11·0; acid-labile P, 7·4%).

Paper-chromatographic and Electrophoretic Data.—Ascending chromatograms on Whatman No. 1 paper were used with solvent systems A, butan-1-ol–acetic acid–water (5 : 2 : 3), and B, *iso*butyric acid–N-ammonia–0·1M-ethylenediaminetetra-acetic acid (100 : 60 : 1·6). The results of electrophoresis in 0·1M-potassium dihydrogen phosphate on Whatman No. 4 paper at 300 v during 8 hr. are tabulated:

	R_F in system A	R_F in system B	Distance travelled (cm.) on electrophoresis
Thymidine-5′ phosphate	0·30	0·42	5·0
Thymidine-5′ pyrophosphate	0·20	0·28	8·4
Thymidine-5′ triphosphate	0·10	0·22	10·0

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UNIVERSITY CHEMICAL LABORATORY, CAMBRIDGE. [*Received, November 26th*, 1957.]